



## Semaphorin 3F forms an anti-angiogenic barrier in outer retina



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### ABSTRACT

**Semaphorins are known modulators of axonal sprouting and angiogenesis. In the retina, we identified a distinct and almost exclusive expression of Semaphorin 3F in the outer layers. Interestingly, these outer retinal layers are physiologically avascular. Using functional *in vitro* models, we report potent anti-angiogenic effects of Semaphorin 3F on both retinal and choroidal vessels. In addition, human retinal pigment epithelium isolates from patients with pathologic neovascularization of the outer retina displayed reduced Semaphorin 3F expression in 10 out of 15 patients. Combined, these results elucidate a functional role for Semaphorin 3F in the outer retina where it acts as a vasorepulsive cue to maintain physiologic avascularity.**

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### 1. Introduction

Semaphorins were originally identified as neuronal guidance cues for axonal pathfinding [1,2]. Most semaphorins are membrane-bound ligands with the exception of soluble class 3 semaphorins [3]. Beyond their role in axonal guidance, semaphorins have been identified as playing pivotal roles in tumor-associated angiogenesis [4]. Their dual function in guiding both neuronal as well as vascular cells renders semaphorins particularly interesting in retina research as the neuro-vascular interface plays a decisive role in both normal retinal development, homeostasis and pathology [5]. Recently, we and others have identified a novel role for class 3 semaphorins in pathologic angiogenesis of the inner retina [6,7].

The inner retina consists of the ganglion cell layer (GCL), the inner plexiform layer (IPL) and the inner nuclear layer (INL) and is supplied with oxygen and nutrients via the three-layered retinal blood vessel plexus [8]. Important examples of neurovascular diseases affecting these inner retinal layers are diabetic retinopathy, retinal vessel occlusion and retinopathy of prematurity [9,10]. In contrast, the deeper layers of the retina are supplied via an entirely different vascular plexus. Both the outer nuclear layer (ONL),

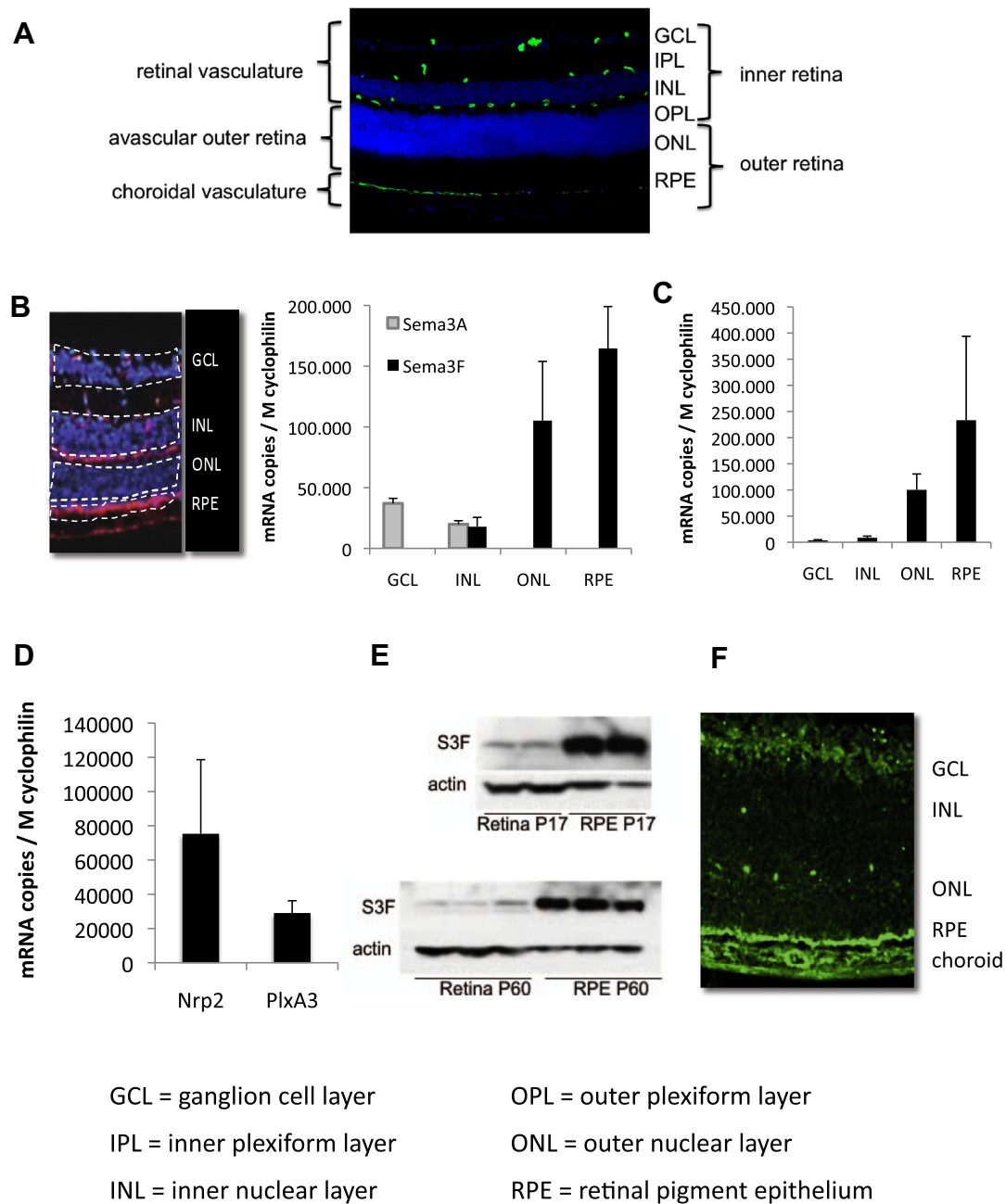
which is formed by the photoreceptor cell nuclei, and the retinal pigment epithelium (RPE) obtain their oxygen and nutrients from choroidal capillaries running directly beneath the neurosensory retina. Importantly, these outer retinal layers themselves are avascular (Fig. 1A).

The exact mechanisms that maintain physiologic avascularity of the outer retina are unknown. Thus, identifying the molecular players that prevent vessels from entering the outer retina may provide valuable insight for designing therapeutic strategies to counter neovascular eye diseases in which the physiologic avascularity of the outer retina is breached. Examples of such disorders include the exudative form of age-related macular degeneration (AMD), in which choroidal capillaries invade the avascular outer retina from below [11]. In other diseases like retinal angiomatous proliferation (RAP), retinal vessels invade the outer retinal layers from above [12]. In both cases, plasma leakage, edema and hemorrhage from pathologic vessels in the outer retina can lead to a rapid decrease in visual acuity.

The aim of this study was to investigate if class 3 semaphorins play a role in upholding the physiologic avascularity of the outer retina by repelling retinal and choroidal capillaries from this region. We first performed a screening analysis of murine semaphorin expression in retina and RPE, followed by validation in specimens from human donor eyes. Our results show a selective

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**Fig. 1.** Physiologic avascularity of the outer retina and expression of Sema3F. (A) The inner retinal layers are supplied by three layers of retinal capillaries (stained green for CD31, blue for cell nuclei). The outer retina consisting of photoreceptors (outer nuclear layer; ONL) and retinal pigment epithelium (RPE) is physiologically void of blood vessels and obtains its oxygen and nutrient supply from the underlying choroidal vasculature. (B) Left: Exemplary retina cross-section demonstrating laser-capture of retinal layers. Right: qPCR quantification of laser-captured layers demonstrating selective expression of Sema3A in ganglion cell layer (GCL) and inner nuclear layer (INL) while Sema3F is expressed selectively and at much higher levels in ONL and RPE of the outer retina ( $n = 3$  retinas; error bars represent SEM). (C) qPCR analysis on laser-captured layers from adult mice (postnatal day 60) confirms selective expression of Sema3F in ONL and RPE of the outer retina ( $n = 3$  retinas; error bars represent S.E.M.). (D) Laser-capture of retinal vessels followed by qPCR reveals expression of the Sema3F receptors Nrp2 and Plexin A2 on retinal endothelial cells in vivo. (E) Western blot analysis confirms high Sema3F levels in RPE at both time points (P17 and P60) on protein level ( $n = 3$  retinas). (F) Immunohistochemistry on murine retina at P17 further confirms localization of Sema3F in the outer retina at the photoreceptor/RPE interface.

expression of Semaphorin 3F (Sema3F) in the outer retinal layers in both mice and humans. Functional experiments demonstrated pronounced anti-angiogenic effects of Sema3F on human retinal endothelial cells and choroidal explant sprouting. Combined, these results provide evidence that Sema3F is expressed selectively in the avascular outer retina and exerts anti-angiogenic effects on retinal and choroidal capillaries.

## 2. Materials and methods

### 2.1. Laser capture microdissection

Eyes were enucleated from C57 BL/6 wildtype mice at indicated time points during normal postnatal development and embedded in optimal cutting temperature (OCT) compound. The eyes were

sectioned at 12  $\mu\text{m}$  in a cryostat, mounted on ribonuclease (RNase)-free polyethylene naphthalate glass slides (11505189, Leica), and immediately stored at  $-80^\circ\text{C}$ . Slides containing frozen sections were fixed in 50% ethanol for 15 s, followed by 30 s in 75% ethanol, before being washed with diethyl pyrocarbonate (DEPC)-treated water for 15 s. Sections were stained with fluoresceinated Isolectin B4 (Alexa Fluor 594, Invitrogen, 1:50 dilution in  $\text{ImM}\text{CaCl}_2$  in PBS) and treated with RNase inhibitor (Roche) at  $25^\circ\text{C}$  for 3 min. Retinal layers were then laser-microdissected with the Leica LMD 6000 system (Leica Microsystems) and collected directly into lysis buffer from the RNeasy Micro kit (Qiagen).

## 2.2. RT-PCR and quantitative real-time PCR analysis

Eyes were rapidly enucleated and whole retinas (or laser-captured neovessels/layers) were processed for RNA extraction using Qiagen columns, followed by treatment with deoxyribonuclease (DNase) I (Qiagen) to remove any contaminating genomic DNA. The DNase-treated RNA was then converted into complementary DNA (cDNA) with reverse transcriptase (Invitrogen). PCR primers were designed with Primer Bank and National Center for Biotechnology Information (NCBI) Primer Blast software. Quantitative analysis of gene expression was generated with an ABI Prism 7700 Sequence Detection System and the SYBR Green Master Mix kit, and gene expression was calculated relative to cyclophilin A expression.

## 2.3. Western blotting

Retinal samples were obtained as described above. Pooled retinal lysate (30 mg) from three different animals was loaded on an SDS–polyacrylamide gel, separated by electrophoresis (SDS–PAGE) and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. After blocking, the membranes were incubated overnight with 1:500 rabbit antibody to mouse Sema3F (Abeam). Membranes were washed and subsequently incubated with 1:2000 horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham) for 1 h at room temperature.

## 2.4. Immunohistochemistry

For immunohistochemistry, eyes were enucleated from mice or human donor post mortem and fixed in 4% paraformaldehyde at room temperature for 1 h. Eyes were then embedded in OCT-compound and sectioned in a cryostat. Primary antibody targeting murine or human Sema3F were from rabbit (Abeam and Millipore, respectively). Secondary fluorescent labeled antibodies were anti-rabbit (Arcis anti rabbit fluorescent secondary antibody).

## 2.5. Microvascular sprouting from retinal endothelial cell spheroids

Human retinal endothelial cells (HRECs) were obtained from Cell Systems and used from passage 2 to 5 for sprouting experiments. Cells were cultured as monolayers at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  in a humidified atmosphere in complete medium (Cell Systems). The preparation of endothelial cell spheroids was performed as described [13,14]. Briefly, cells were harvested from subconfluent monolayers by trypsinization and suspended in complete medium containing 10% fetal bovine serum (FBS) and 0.25% (w/v) carboxymethylcellulose (Sigma). Five hundred cells were seeded together in one hanging drop to assemble into a single spheroid within 24 h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . After 24 h, spheroids were harvested and used for sprouting analysis in a matrix of type I collagen. Briefly, 30 endothelial cell spheroids per group were seeded into 0.5 ml of collagen solution in non-adherent 24-well plates, with a final concentration of rat type I collagen of 1.5 mg/ml. Freshly

prepared gels were transferred rapidly into a humidified incubator ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ), and after the gels had solidified, 0.1 ml of serum-free medium (Cell Systems) was added per well containing VEGF  $\pm$  Sema3F recombinant protein (RnD Systems). After 24 h, gels were photomicrographed and spheroid sprouting assessed quantitatively using Adobe Photoshop. Results are expressed as means  $\pm$  S.E.M.

## 2.6. Microvascular sprouting from choroidal explants

Choroidal explant experiments were performed similar to previously described experiments [15]. Briefly, eyes from normal C57BL/6 adult wildtype mice were enucleated and immediately processed for isolation of the RPE/choroid compartment. Equally sized pieces were placed in a matrix of type I collagen and incubated in  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  for 72 h. All explants were stimulated with VEGF-A (20 ng/ml)  $\pm$  Sema3F recombinant protein. Photographs of individual explants were taken and the area covered by microvascular sprouting was quantified using digital imaging software analysis.

## 2.7. CNV-RPE

CNV membranes had been obtained from patients in a time before intravitreal anti-VEGF injections became the gold standard for treating exudative AMD and were characterized before [16]. In brief, CNV membranes had been surgically excised and RPE was cultivated from these membranes. All CNV RPE cells were isolated using the same protocol. Care was taken to use lowest possible passage numbers. However, some passaging of cells was necessary in order to yield sufficiently clean RPE cultures. The isolation and passaging protocol is outlined in detail in Schlunck et al. (2002) [16]. Control RPE cells were isolated from a healthy donor eye, not a CNV membrane, but otherwise cultured and treated in the same way as CNV RPE. For measuring Sema3F expression, we had 15 CNV-RPE RNA aliquots from 15 different AMD patients available. For additional experiments investigating other angiogenesis-associated genes, 10 samples were left for analysis. Reverse transcription and qPCR were performed as described above. Results are presented normalized to actin expression. Ratios were calculated using the  $\Delta\Delta\text{Ct}$  method.

## 3. Results and discussion

Based on our hypothesis that vasorepellent class 3 semaphorins play a role in upholding physiologic avascularity in the outer retina, we first performed an expression analysis of different class 3 semaphorins in murine retina using laser-capture microdissection followed by qPCR (Fig. 1B). Both Sema3A and Sema3F were expressed in the murine retina. For Sema3B, 3C and 3D we did not detect replicable expression levels in retinal laser-capture samples. Interestingly, Sema3A and Sema3F showed a very distinct and non-overlapping distribution pattern in the murine retina. Sema3A was exclusively found in the inner retina (ganglion cell layer and inner nuclear layer). Sema3F, in contrast, was predominantly expressed in the outer retina with most robust expression in the RPE layer. For Sema3A, expression in the inner retina is in line with earlier data from our group showing pronounced expression of Sema3A in hypoxic ganglion cells of the inner retina secondary to oxygen-induced retinopathy (OIR) [6]. In contrast to the hypoxic situation in the OIR model, Sema3A expression in ganglion cells during normal development is relatively low. However, physiologic Sema3F expression in the outer retina is significantly higher, suggesting a housekeeping role for Sema3F but not Sema3A during normal tissue homeostasis of the outer retina (Fig. 1B). The laser-capture

expression profile for Sema3F in the developing mouse retina was replicated in independent experiments using adult mice at P60 (Fig. 1C).

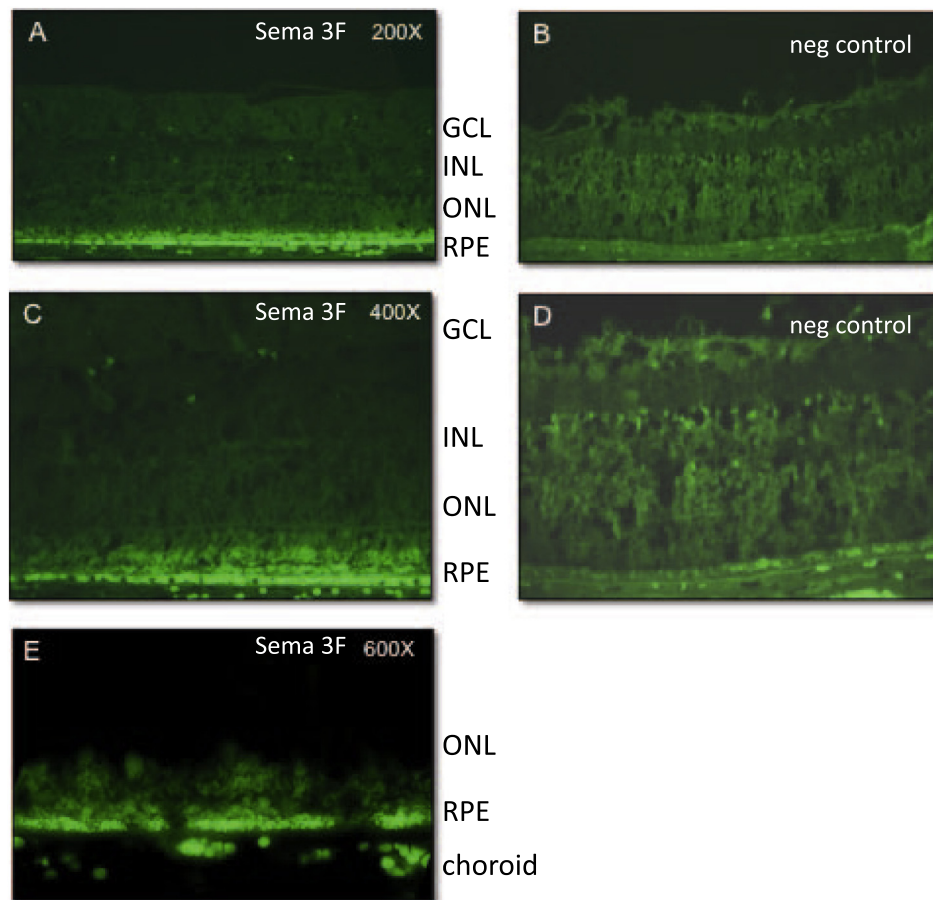
Importantly, the receptors for Sema3F, neuropilin 2 (Nrp2) [17] and PlexinA3 [18], are both expressed on retinal endothelial cells *in vivo* as confirmed by laser-capture microdissection (Fig. 1D). Sema3F is thus able to bind and signal in retinal endothelial cells, exerting its angiorepellent effect. Selective Sema3F mRNA expression in the outer retina was verified using Western blot protein expression analysis in both developing and adult mice (Fig. 1E). Consistent with results from qPCR, Sema3F was consistently expressed at its highest levels in the RPE layer. These quantitative data were further confirmed by immunohistochemical analysis that localized Sema3F at the outer photoreceptor/RPE interface (Fig. 1F).

Additionally, the murine expression data were tested for their potential translational impact with the analysis of Sema3F expression in human retina from a healthy donor eye (Fig. 2). Consistent with the murine expression results, Sema3F in normal human retina localized to the outermost retinal layers and RPE (Fig. 2A, C, and E). Negative controls without primary antibody showed only slight background fluorescence from blood cells trapped in choroidal capillaries (Fig. 2B and D).

We next investigated whether Sema3F might play a functional role in upholding the physiologic avascularity of the outer retina. To this end, one would expect Sema3F to exert anti-angiogenic functions on both retinal as well as choroidal capillaries to prevent

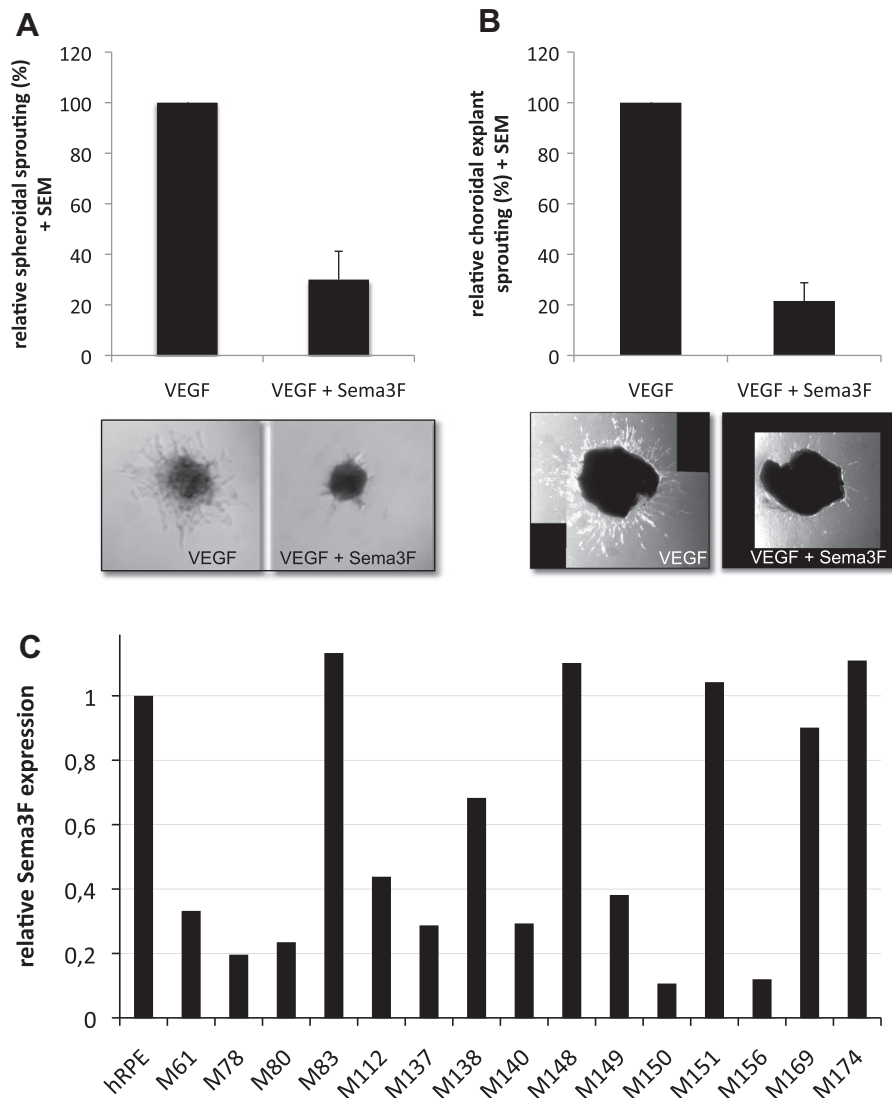
them from invading into the avascular outer retina from either above (retinal vessels) or below (choroidal vessels). First we explored the effect of Sema3F on retinal endothelial cells using a 3D *in vitro* model in which human retinal endothelial cells (HRECs) were cultivated as spheroidal cell aggregates and placed in a collagen matrix. Upon angiogenic stimulation with VEGF, HREC spheroids sprout into the surrounding matrix; addition of Sema3F significantly reduced this VEGF-induced HREC sprouting. These results identify an anti-angiogenic effect of Sema3F on retinal endothelial cells (Fig. 3A). Using a second functional approach, we tested the effect of Sema3F on choroidal neovascularization in an *in vitro* sprouting model of choroidal explants (CEs). Upon stimulation with VEGF, CE sprout into the surrounding matrix protruding extensions of choroidal capillaries. Addition of Sema3F to VEGF-stimulated CE significantly reduced explant sprouting, confirming the anti-angiogenic properties of Sema3F on choroidal endothelial cells (Fig. 3B). Combined, these functional experiments demonstrate a pronounced anti-angiogenic effect of Sema3F on both retinal as well as choroidal endothelial cells.

If this anti-angiogenic role of Sema3F played a functional role *in vivo*, one would expect reduced Sema3F expression in patients with pathologic neovascularization of the outer retina. We therefore investigated Sema3F expression in RPE cells isolated from patients with choroidal neovascularization (CNV) in exudative AMD. In these patients, the physiologic anti-angiogenic barrier in the outer retina is breached and pathologic choroidal vessels invade into the outer retina. The RPE cells from CNV membranes were



**Fig. 2.** Sema3F expression in normal human retina. (A, C, E) In analogy to the results seen in murine eyes, Sema3F localizes to the outer retinal border at the photoreceptor/RPE interface in human retinas. Images show representative samples at three different magnifications. (B, D) Negative controls without primary antibody demonstrate only weak background fluorescence. Note the complete lack of the specific Sema3F hyperfluorescent band at the photoreceptor/RPE interface despite increased exposure time in negative control images. Some red blood cells trapped in choroidal capillaries display autofluorescence in the negative control images.





**Fig. 3.** Sema3F inhibits angiogenic sprouting of both retinal and choroidal endothelial cells. (A) Human retinal endothelial cells (HRECs) cultivated as multicellular spheroids sprout into the surrounding collagen matrix when stimulated with VEGF (25 ng/ml; set to 100%). Addition of Sema3F (900 ng/ml) significantly reduces angiogenic sprouting of VEGF-stimulated HREC-spheroids.  $p = 0.003$ ,  $n = 5$ . (B) VEGF-induced angiogenic sprouting from murine choroidal explants is similarly reduced by recombinant Sema3F.  $p = 0.002$ ,  $n = 4$ . (C) Relative expression of Sema3F in RPE isolated from a healthy human donor eye (hrPE; set to 1) and CNV-RPE isolated from 15 different patients with choroidal neovascularization (CNV) in exudative age-related macular degeneration (AMD). Numbers M61 to M174 refer to individual patient numbers. In 10 out of 15 CNV-RPE isolates Sema3F expression was reduced compared to hrPE.

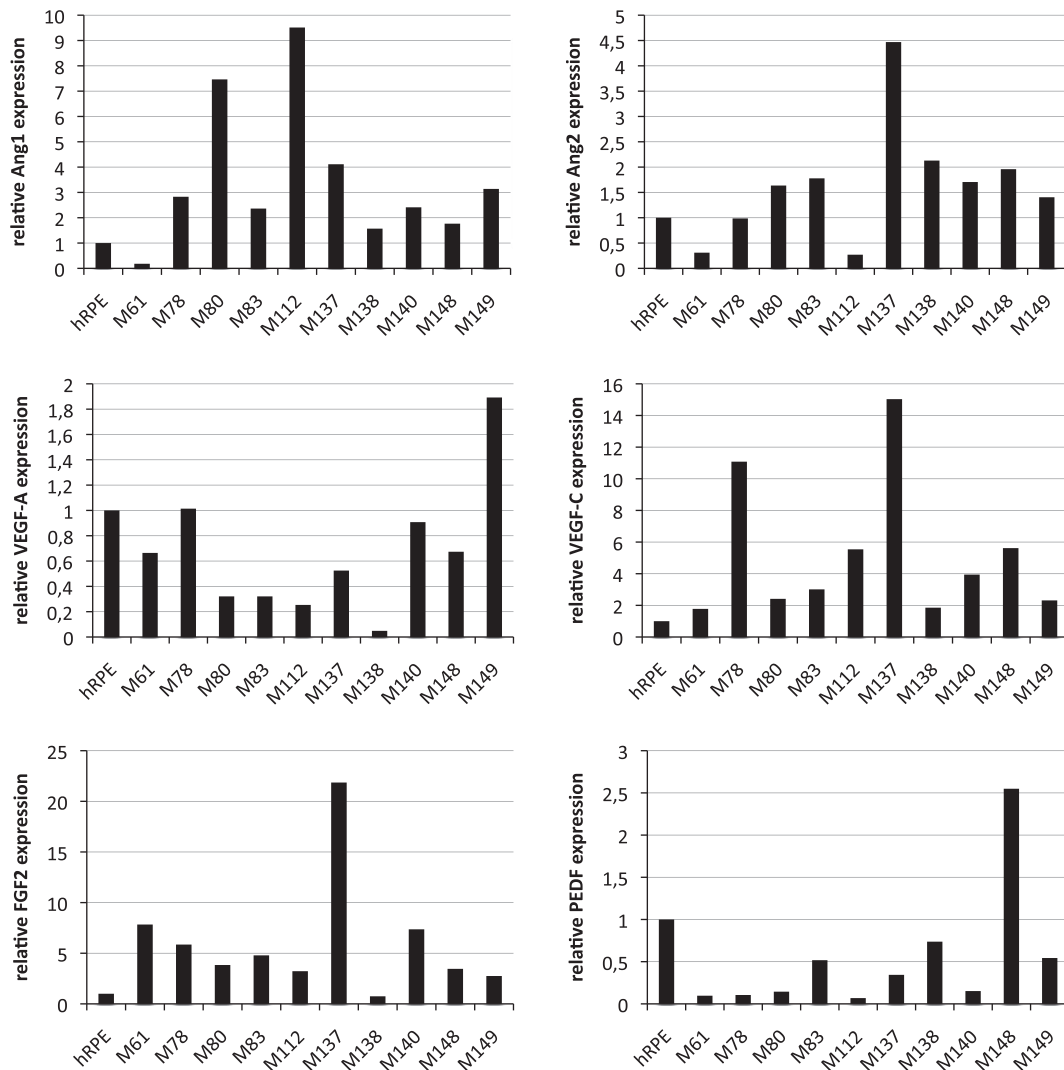
obtained from surgically excised CNV membranes at a date before intravitreal anti-VEGF injections became the gold standard for treating exudative AMD and were characterized before [16]. Our results show that in 10 out of 15 CNV-RPE isolates Sema3F expression was indeed reduced compared to human RPE from a healthy donor eye (hrPE; Fig. 3C). While these qPCR results can only serve as an indication, they suggest that reduced Sema3F expression plays a role in the breakdown of the anti-angiogenic barrier in the outer retina of some (but not all) patients with exudative AMD. In addition, further experiments revealed that changes in Sema3F expression were not consistently associated with changes in other known angiomodulatory factors, thus suggesting an independent angiomodulatory role for Sema3F (Fig. 4).

In summary, our data demonstrate a distinct expression pattern for Sema3F in outer retina and RPE that is consistent over time and may play a role in maintaining outer-retinal avascularity. Functionally, both retinal as well as choroidal endothelial cell growth is inhibited by Sema3F. These results render Sema3F a promising candidate to investigate further as a potentially important player

in regulating physiologic outer retina avascularity. Clinically, changes in Sema3F expression or function may play a role in diseases like wet AMD where the avascularity of the outer retina is breached and choroidal neovessels invade into the subretinal space. Therapeutically, Sema3F may provide a natural endogenous natural inhibitor against pathological chorioretinal neovascularization in these patients.

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**Fig. 4.** Relative expression of a panel of angiogenesis-related genes in RPE isolated from a healthy human donor eye (hRPE; set to 1) and CNV-RPE. Angiopoietin 1 (Ang-1), angiopoietin 2 (Ang-2), VEGF-A, VEGF-C, fibroblast growth factor 2 (FGF2) and pigment epithelium derived factor (PEDF) are all expressed differentially in isolated CNV-RPE samples. However, expression of these factors is not directly linked to Sema3F expression changes displayed in Fig. 3C. These findings suggest that Sema3F expression changes are not secondary to changes in one of these known angiomodulatory factors.

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